Identification of the Major Urinary Metabolite of the F_2-isoprostane 8-Iso-prostaglandin $F_{2\alpha}$ in Humans*

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F_2-isoprostanes are prostaglandin-like products of nonenzymatic lipid peroxidation. Measurement of levels of endogenous unmetabolized F_2-isoprostanes has proven to be a valuable approach to assess oxidative stress *in vivo*. However, measurement of levels of urinary metabolites of F_2-isoprostanes in timed urine collections offers an advantage over measuring unmetabolized F_2-isoprostanes, e.g. in a plasma sample, in that it can provide an integrated index of isoprostane production over time. Therefore, we sought to identify the major urinary metabolite in humans of one of the more abundant F_2-isoprostanes produced, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-PGF$_{2\alpha}$). 20 μCi of tritiated 8-iso-PGF$_{2\alpha}$ was infused over 1 h into a male volunteer. 75% of the infused radioactivity was excreted into the urine during the following 4.5 h and was combined with urine collected for 4 h from a rhesus monkey following infusion of 500 μg of unlabeled 8-iso-PGF$_{2\alpha}$. Urinary metabolites were isolated and purified by adsorption chromatography and high pressure liquid chromatography. The major urinary metabolite, representing 29% of the total extractable recovered radioactivity in the urine, was structurally identified by gas chromatography and mass spectrometry as 2,3-dinor-5,6-dihydro-8-iso-prostaglandin $F_{2\alpha}$. The identification of 2,3-dinor-5,6-dihydro-prostaglandin $F_{2\alpha}$ as the major urinary metabolite of 8-iso-prostaglandin $F_{2\alpha}$ provides the basis for the development of methods of assay for its quantification as a means to obtain an integrated assessment of oxidative stress status in humans.

Free radicals have been implicated in the pathogenesis of a wide variety of human disorders (1–4). One of the major targets of free radical injury are lipids, which undergo peroxidation. Previously, we reported the discovery that a series of prostaglandin (PG)[1] F_2-like compounds (F_2-isoprostanes (F_2-IPs)) are produced *in vivo* as products of the free radical-catalyzed peroxidation of arachidonic acid (5). Formation of these compounds occurs independently of the cyclooxygenase enzyme and proceeds through intermediates comprising arachidonoyl peroxyl radical isomers of arachidonic acid, which undergo endocyclization to form bicyclic endoperoxides. The endoperoxides are then reduced to yield F_2-IPs. The endoperoxides also undergo rearrangement *in vivo* to form D- and E-ring IPs (6). Four positional isomers of IPs are formed, each of which can comprise eight racemic diastereomers. IPs are initially formed esterified to phospholipids and subsequently released preformed (7). Based on the mechanism of formation of IPs, *i.e.* the formation of compounds with the side chains oriented *cis* in relation to the cyclopentane ring are highly favored (8), one compound that would be expected to be formed would be 8-iso-PGF$_{2\alpha}$. Recently we demonstrated that 8-iso-PGF$_{2\alpha}$ is in fact one of the more abundant F_2-IPs produced *in vivo* (9). There has been considerable interest in this molecule, because it exerts biological activity, e.g. it is a potent vasoconstrictor in the lung and kidney (10, 11). Furthermore, it has been suggested that the biological effects of 8-iso-PGF$_{2\alpha}$ may result from an interaction with a unique receptor (12).

It has been recognized that one of the greatest impediments in the field of free radical research has been the lack of reliable methods to assess oxidant stress status in humans (13). A considerable body of evidence has accumulated indicating that measurement of F_2-IPs provides a valuable and reliable approach to assess oxidant stress *in vivo* both in animal models of oxidant injury and in humans (14, 15). In this regard, however, quantification of unmetabolized IPs has certain limitations. First, F_2-IPs can be artifactually generated *ex vivo*, e.g. in plasma, by auto-oxidation of plasma arachidonic acid if appropriate precautions are not taken (8). In addition, quantification of F_2-IPs esterified in tissues or circulating in plasma only provides information at a single point in time rather than an integrated index of IP production. Having a means to obtain an integrated index of oxidant stress status would be very valuable in situations in which the level of oxidant stress fluctuates over time. In this regard, analogous to quantification of urinary metabolites of cyclooxygenase-derived prostanoids (16), measurement of the urinary excretion of F_2-IPs should provide a reliable and integrated index of oxidative stress status *in vivo*.

We have previously identified urinary metabolites of F_2-IPs that copurify through a mass spectrometric assay developed for quantification of the major urinary metabolite of cyclooxygenase-derived PGD$_2$ (17, 18). However, we do not know the parent compounds from which these F_2-IP metabolites derive. Furthermore, we have found that a metabolite of cyclooxygenase-derived PGF$_{2\alpha}$, 9α,11α-dihydroxy-15-oxo-13,14-dihydro-2,3,18,19-tetranorprost-1,20-dioic acid, cochromatographs on capillary gas chromatography (GC) with these F_2-IP metabolites.[2] This latter finding confounds an interpretation as to whether

[1] The abbreviations used are: PG, prostaglandin; F_2-IP, F_2-isoprostane; GC, gas chromatography; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

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an increase in the intensity of these peaks when analyzed by GC and mass spectrometry (MS) represents overproduction of F_2-IPs or $PGF_{2\alpha}$. Thus, we undertook a study to identify the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ in humans as a basis for the development of methods of assay for its quantification to assess oxidative stress status in humans.

EXPERIMENTAL PROCEDURES

Reagents – Unlabeled 8-iso-$PGF_{2\alpha}$ was obtained from Cayman Chemical (Ann Arbor, MI). [³H]8-iso-$PGF_{2\alpha}$ (50 Ci/mmol) was commercially prepared from unlabeled 8-iso-$PGF_{2\alpha}$ by SibTek Inc. (Tenafly, NJ) as a randomly labeled compound. Compound purity and specific activity of the [³H]8-iso-$PGF_{2\alpha}$ were confirmed by GC and MS. Amberlite XAD-2 resin and silicic acid (mesh size, 100–200) were obtained from Sigma. All organic reagents were purchased from Baxter (Burdick and Jackson Brand, McGaw Park, IL). Pentafluorobenzyl bromide and diisopropylethylamine were obtained from Aldrich. [²H₉]N,O-bis(trimethylsilyl)trifluoroacetamide was purchased from Regis Chemical Co. (Morton Grove, IL). 1-Butaneboronic acid was obtained from Applied Science Laboratories (State College, PA).

Experimental Strategy for Determining the Metabolic Fate of 8-iso-$PGF_{2\alpha}$ in Humans – Because 8-iso-$PGF_{2\alpha}$ exerts potent biological activity, we used a strategy whereby only a tracer quantity of 8-iso-$PGF_{2\alpha}$ was infused into a human and 500 µg of unlabeled 8-iso-$PGF_{2\alpha}$ was infused into a monkey. Urine specimens collected from the human and monkey following these infusions were then combined. Using this approach, the relative abundance of the various metabolites reflected by radiolabeled peaks on chromatographic purification would reflect what occurs in humans, whereas the amount of unlabeled material required for structural identification would be derived from the monkey. Although the metabolism of prostanoids in the monkey closely mimics that in humans (16, 19), the approach we used would eliminate any ambiguity about extrapolating data obtained from determining the metabolic fate of 8-iso-$PGF_{2\alpha}$ in a monkey to that in humans.

Infusion of [³H]8-iso-$PGF_{2\alpha}$ into a Human Volunteer – After informed consent was obtained, 20 µCi of [³H]8-iso-$PGF_{2\alpha}$ was infused over 1 h in 50 ml of sterile normal saline into an antecubital vein of a normal volunteer. Urine was collected from the beginning of the infusion until 6 h after the infusion and stored at –70 °C until processed.

Infusion of 8-iso-$PGF_{2\alpha}$ into a Monkey – 500 µg of unlabeled 8-iso-$PGF_{2\alpha}$ combined with 0.6 µCi of [³H]8-iso-$PGF_{2\alpha}$ was resuspended in 200 ml of normal saline sterile and infused into the superficial femoral vein of a 10-kg rhesus monkey over 2 h. The small quantity of radiolabeled 8-iso-$PGF_{2\alpha}$, which represented only 3% of the amount of radiolabeled 8-iso-$PGF_{2\alpha}$ infused into the human, was infused along with the unlabeled 8-iso-$PGF_{2\alpha}$ to monitor the time course of excretion of metabolites into the monkey urine. Prior to the procedure, the animal was anesthetized with halothane and remained under anesthesia until the infusion was completed. After infusion, urine was collected for 6 h in a specially designed cage that separates urine from feces. The protocol was approved by the Vanderbilt University Animal Care Committee.

Extraction and Adsorption Chromatography – Initial extraction of urine was performed using Amberlite XAD-2. XAD-2 was suspended in distilled water, and a column (8-cm inside diameter) was packed by sedimentation to a final size of approximately 750 ml. Pooled urine samples (approximately 2000 ml) from both the human and monkey were combined, acidified to pH 3 with 1 N HCl, and percolated through the column of XAD-2. The column was then washed with 1500 ml of H_2O (pH 3), and the radioactivity was eluted with ethanol in 8 × 100-ml fractions. The ethanol eluates containing significant amounts of radioactivity were then evaporated under reduced pressure. The residue was resuspended in 50 ml of phosphate-buffered saline (pH 7.4), acidified with 1 N HCl to pH 3, and extracted three times with 50 ml of ethyl acetate. The ethyl acetate extracts were combined and applied to a 25-g column (3.2-cm inside diameter) of silicic acid, and radioactivity was eluted with 400 ml of ethyl acetate.

Separation and Purification of 8-iso-$PGF_{2\alpha}$ Metabolites by High Pressure Liquid Chromatography (HPLC) – The ethyl acetate eluate from the silicic acid column was evaporated under reduced pressure, and the residue was then subjected to normal phase HPLC using a 5-µm 30-cm × 10-mm Adsorbosphere silica column (Alltech, Deerfield, IL) using a gradient solvent system with linear programming of chloroform/acetic acid (100:0.1) to chloroform/methanol/acetic acid (90:10:0.1) over 3 h at a flow rate of 4 ml/min. The major radioactive peak eluted was then subjected to reversed phase HPLC using a 5-µm 25-cm × 4.6-mm Econosil C18 column (Alltech) with an isocratic solvent system of water/acetonitrile/acetic acid (80:20:0.1) at a flow rate of 1 ml/min. The single

radioactive peak that eluted was then converted to a methyl ester with ethereal diazomethane and rechromatographed on reversed phase HPLC using the same column noted above with a mobile phase of water/acetonitrile (80:20) at a flow rate of 1 ml/min.

Mass Spectrometric Analysis of Major Urinary Metabolite of 8-iso-$PGF_{2\alpha}$ – The major urinary metabolite of 8-iso-$PGF_{2\alpha}$ was analyzed by GC-negative ion chemical ionization-MS and by electron ionization-MS. For negative ion chemical ionization analysis, the compound was converted to the pentafluorobenzyl ester trimethylsilyl ether derivative. Catalytic hydrogenation was performed as described previously (8). Analysis was performed on a Nermag R10-10C mass spectrometer interfaced with a DEC-PDP computer. GC was carried out using a 15-m, 0.25-µm film thickness, DB-1701 fused silica capillary column (J & W Scientific, Folsom, CA) as described (8). Electron ionization-MS of the methyl ester trimethylsilyl ether derivative of the metabolite was carried out as described previously using a Finnigan Incos 50 mass spectrometer (8).

RESULTS AND DISCUSSION

Infusions of 8-iso-$PGF_{2\alpha}$ – The infusions of 8-iso-$PGF_{2\alpha}$ into the human volunteer and the monkey were not associated with any significant changes in blood pressure or pulse rate, and no clinically apparent adverse effects were observed. 75% of the total radioactivity infused in the human was recovered in the urine in 4.5 h, and 95% of the radioactivity infused into the monkey was recovered in the urine in 4 h. Urine specimens from both the monkey and human were then combined for isolation and purification of metabolites.

Extraction and Adsorption Chromatography of 8-iso-$PGF_{2\alpha}$ Metabolites – Initial compound isolation was achieved by using Amberlite XAD-2 resin chromatography. After loading the sample and washing the column, compounds were eluted with 8 × 100-ml aliquots of ethanol. 98% of the radioactivity was present in aliquots 5–7. Subsequently, radioactive material eluting in these fractions was evaporated and resuspended in ethyl acetate for adsorption chromatography on silicic acid. It was found, however, that a significant portion of the radioactivity (approximately one-half) was insoluble in ethyl acetate. In contrast, all of the radioactivity was soluble in phosphate-buffered saline (pH 7.4). Thus, after resuspension in buffer, the aqueous phase was acidified to pH 3 and extracted with ethyl acetate. 58% of the radioactivity extracted into the organic phase, but 42% remained in the aqueous phase, even after exhaustive extractions with ethyl acetate. This suggested that the unextractable metabolites were highly polar, perhaps in the form of a polar conjugate (20). Work is currently underway to identify the nature of these highly polar compounds.

The material that extracted into ethyl acetate was then applied to a column of silicic acid, and 95% of the applied radioactivity eluted with 400 ml of ethyl acetate.

HPLC Isolation and Purification of 8-iso-$PGF_{2\alpha}$ Metabolites – Radioactive material eluting from the silicic acid column was initially subjected to normal phase HPLC, as described under "Experimental Procedures." The chromatogram obtained is shown in Fig. 1. As is evident, the vast majority of radioactivity eluted within the first 90 min and multiple radioactive peaks are present. However, there was a single major peak (*) that eluted between 65 and 69 min. Material in this peak was then subjected to further purification as a free acid on reversed phase HPLC using an isocratic solvent system of water/acetonitrile/acetic acid (80:20:0.1). As shown in Fig. 2, essentially all of the recovered radioactivity (>95%) eluted as a single peak between 56 and 60 min.

Material in this peak was then converted to a methyl ester and rechromatographed on reversed phase HPLC using a solvent system of water/acetonitrile (80:20). Virtually all the radioactivity (>95%) eluted as a single peak between 27 and 31 min. The fact that the single prominent radioactive peak that eluted between 65 and 69 min on the initial normal phase
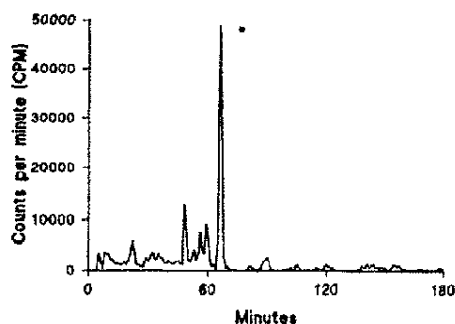
FIG. 1. Normal phase HPLC analysis of urinary metabolites following extraction of urine and purification by adsorption chromatography. A single major peak (*) eluted between 65 and 69 min.
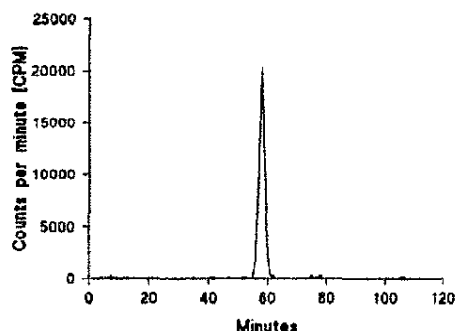

FIG. 2. Reversed phase HPLC analysis of material that eluted between 65 and 69 min in Fig. 1. Essentially all of the radioactivity applied eluted in a single peak between 56 and 60 min.

HPLC was found to elute as a single sharp peak on the two subsequent reversed phase HPLC purification steps suggested that this was a single compound and represented the major urinary metabolite of 8-iso-$PGF_{2\alpha}$. This compound comprised 29% of the total recovered extractable radioactivity present in the urine.

Mass Spectrometric Analysis of the Major Urinary Metabolite of 8-iso-$PGF_{2\alpha}$—This major metabolite was then analyzed by both electron ionization-MS and negative ion chemical ionization-MS. A portion was converted to a methyl ester, trimethylsilyl ether ether derivative, and analyzed by electron ionization-MS. The mass spectrum obtained for this compound is shown in Fig. 3. A prominent molecular ion was present at m/z 558. Additional prominent ions were also present at m/z 543 (M − 15, loss of ˙CH_3); m/z 487 (M − 71, loss of ˙CH_2-$(CH_2)_3$-CH_3); m/z 468 (M − 90, loss of Me_3SiOH); m/z 453 (M − 90 − 15); m/z 437 (M − 90 − 31, loss of 90 + ˙OCH_3); m/z 397 (M − 90 − 71); m/z 378 (M − (2 × 90)); m/z 313 (M − 199 − 31 − 15, loss of ˙$(CH_2)_2$-$CHOSiMe_3$-$(CH_2)_4$-CH_3 + 31 + 15); m/z 307 (M − (2 × 90) + 71); m/z 281 (M − 186 − 90 − H, loss of ˙CH_2-$CH(OSiMe_3)$-$(CH_2)_4$-CH_3 + 90); m/z 217 (Me_3SiO-$CH=CH$-$CH=^+OSiMe_3$); m/z 199 ($^+CH=CH$-$CH(OSiMe_3)$-$(CH_2)_4$-CH_3); m/z 191 ($Me_3SiO^+=CH$-$OSiMe_3$), a rearrangement ion characteristic of F-ring prostanoids (8); m/z 173 ($Me_3SiO^+=CH$-$(CH_2)_4$-CH_3); m/z 147, and m/z 129 ($Me_3SiO^+=CH$-$CH=CH_2$). On the basis of this mass spectrum, this metabolite was identified as 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. In the mass spectrometric analysis of other eicosanoids, the loss of 186 + H from the molecular ion has been noted to occur with fragmentation across the Δ^{13} double bond (21–23). The ion at m/z 199 is a typical ion present in the mass spectra of both $PGF_{2\alpha}$ and 8-iso$PGF_{2\alpha}$ and represents the lower side
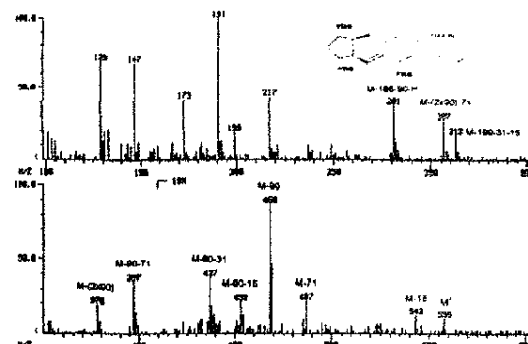

FIG. 3. Electron ionization mass spectrum of the methyl ester trimethylsilyl ether derivative of the major urinary metabolite of 8-iso-$PGF_{2\alpha}$.

chain from C13 to C20 (22). The presence of this ion was important in that it indicated that the Δ^{13} double bond was intact, and thus it was the Δ^5 double bond that has been reduced. It is of interest that the Δ^5 double bond is reduced in this metabolite, which is major metabolite of 8-iso-$PGF_{2\alpha}$. In previous metabolism studies of other prostanoids and thromboxane B_2 in nonhuman primates and humans, only very minor metabolites of thromboxane B_2 have been identified in which the Δ^5 double bond had been reduced (23). One might speculate that inversion of the upper side chain stereochemistry in 8-iso-$PGF_{2\alpha}$ might render it or 2,3-dinor-8-iso-$PGF_{2\alpha}$ a better substrate for the reductase that reduces the Δ^5 double bond (24).

Additional approaches were undertaken to further confirm the identity of this metabolite of 8-iso-$PGF_{2\alpha}$ as 2,3 dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. First, analysis of the metabolite as a pentafluorobenzyl ester, trimethylsilyl ether derivative by negative ion chemical ionization-MS generated a major fragment ion of 543 Da, representing the expected M − 181 ion (loss of ˙$CH_2C_6F_5$), as would be expected. Second, analysis of the compound as a $[^2H_9]$trimethylsilyl ether derivative resulted in a shift of the m/z 543 peak to greater than 27 Da, indicating the presence of three hydroxyl groups. Third, when the compound was analyzed following catalytic hydrogenation, there was disappearance of the m/z 543 peak and the appearance of a new intense peak 2 Da higher at m/z 545, indicating that the compound contained a single double bond. Finally, analysis of the compound after reaction with 1-butaneboronic acid resulted in the disappearance of the m/z 543 ion and the appearance of a major ion at m/z 465, indicating the formation of a cyclic boronate derivative with the *cis*-cyclopentane ring hydroxyls. Collectively, these results provided additional confirmatory evidence that the metabolite contained the functional groups and the number of double bonds predicted for 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$.

In summary this study has determined that the major urinary metabolite of 8-iso-$PGF_{2\alpha}$ in humans is the product of a single step of β oxidation and reduction of the Δ^5 double bond, resulting in the formation of 2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$. Identification of the major urinary metabolite of the F_2-isoprostane, 8-iso-$PGF_{2\alpha}$, provides the basis for the development of methods of assay for its measurement to obtain an integrated assessment of oxidative stress status *in vivo* in humans over time.

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